Abnormalities in the psoriatic non-lesional skin

Ph.D. Thesis

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1. INTRODUCTION

1.1. Psoriasis, a complex inflammatory skin disease

Psoriasis is a multifactorial skin disorder affecting about 2-3% of the population world-wide, although it is more prevalent in European, American and Canadian populations. The most common form of chronic plaque psoriasis is Psoriasis vulgaris, which is characterized by epidermal hyperplasia, intensified and abnormal dermal angiogenesis and infiltration of inflammatory cells. Altered cellular composition characterized by hyperproliferation of keratinocytes and massive infiltration of immune cells is initiated by secreted cytokines of activated resident immune cells, dendritic cells, macrophages and innate immune cells as well as by keratinocytes themselves in the psoriatic skin. Both genetic risk factors and environmental triggers are known to contribute to the pathomechanism of psoriasis.

Several studies focused mainly on the lesional skin and as a result of these efforts now it is well characterized that psoriasis is a chronic, inflammatory skin disease in which both innate and adaptive immune activation play a role in the manifestation and the maintenance of the abnormal skin phenotype. However, much less is known about the contribution of resident cells of the tissue to the pathology of the disease. Therefore, we focused on to seek for alterations in tissue responses in psoriasis by investigating the phenotypically normal looking non-lesional (NL) psoriatic skin.

1.2. The role of fibronectin-binding integrins

Integrins are cell-surface heterodimeric transmembrane receptors consisting of an α and a β subunit known to play essential roles in connecting the intracellular cytoskeleton with various components of the extracellular matrix (ECM). Fibronectin (FN) is a protein secreted as a soluble dimer and through interactions with integrins they form fibrillar structures. The main FN receptor $\alpha_5\beta_1$ integrin was found to be overexpressed in the NL psoriatic epidermis relative to normal. A possible explanation of α_5 integrin overexpression in NL skin is the presence of FN and one of its isoforms, the extra-domain-A containing fibronectin (EDA^+FN) in the microenvironment of basal keratinocytes.

1.3. Structure and function of EDA^{+}FN and FN

FN is a multifunctional component of the ECM. Matrix FN provide a base to the cells and regulate the cellular behavior through the transfer of biochemical and mechanical signals. There are two major distinct forms of FN: the plasma and the cellular FN. FN could contain one or two extra Type III domains, named *E*xtra *D*omain *A* and *B* (EDA and EDB, respectively). These two domains could be inserted/excluded through alternative splicing. The cellular form of FN contains the EDA domain and found incorporated into the ECM of tissues. The production of alternatively spliced forms is the highest during embryonic development. Postnatal the amount of the protein is reduced and further decreases with ageing. However, elevated amount of the EDA^{+}FN is observed during tissue repair, wound healing, angiogenesis and in highly proliferating adult tissues, it can influence cell adhesion, differentiation, cell cycle progression.

1.4. Structure and function of KGF and FGFR2

Fibroblast growth factors (FGFs) are small growth factor polypeptides. The family of FGF consists of 23 members with a common structural characteristic. Fibroblast growth factor 7 (FGF7), also known as keratinocyte growth factor (KGF) is produced by cells of mesenchymal origin and considered to be a major growth factor for keratinocytes. In the skin KGF is produced by dermal fibroblasts and keratinocytes can influence its production through interleukin 1 (IL-1). KGF is known to induce keratinocyte proliferation and differentiation. There is also evidence suggesting that fibroblasts from L and NL skin of psoriatic patients induce keratinocyte outgrowth by producing soluble signals. Indirect evidence, such as measurement of mitogenic activity, suggests that the specific receptor for KGF on epithelial cells is the IIIb splice variant of fibroblast growth factor receptor 2 (FGFR2).

The FGFR family consist of FGFR1, FGFR2, FGFR3 and FGFR4 encoding for receptors of FGFs. FGFRs are transmembrane-type receptors, composed of an extracellular ligand-binding domain, a single transmembrane domain and a cytoplasmic domain. FGFR2 has two isoforms FGFR2-IIIb and FGFR2-IIIc. KGF is known to initiate mitogenic activity most effectively by binding to the FGFR2-IIIb splice variant. KGF can also facilitate cell proliferation through FGFR2-IIIc or FGFR1, but with a much lower efficiency.

1.5. FGF and FGFR signaling network

FGFs transmit their signal through binding to specific FGFRs. FGFs are secreted glycoproteins that are localized to the extracellular matrix and to the cell surface by heparin sulphate proteoglycans (HPSGs). Activated FGFR2 phosphorylates the FGFR substrate 2 (FRS2) to recruit the son of sevenless and growth factor receptor-bound protein 2 (GRB2) leading to the activation of the RAS-RAF-MEK-MAPK/ERK pathway. AKT-dependent anti-apoptotic signalling cascade is activated by GRB2-associated binding protein 1- PI3K (GAB1-PI3K) in a separate complex. On the other hand, the Src homology 2 (SH2) domain of PLCγ binds and activate the protein kinase C (PKC), which reinforces the activation of MAPK. Finally, the activation of the signal transducer and activator of transcription (STAT) pathway depends on cellular context.

1.6. Roles of KGF and FGFR2 in pathophysiology and therapy

There are several pieces of evidence highlighting the important function of KGF in the processes of wound healing, tissue regeneration and repair. The paracrine signaling functions of KGF are explored for their therapeutic potential in mucositis and wound healing. Genetic alterations of FGFR2 that cause dysregulation of FGFR2 signaling can lead to the development of congenital disorders and acquired diseases including melanoma, Apert syndrome and atopic dermatitis among others. The FGFR2 gene is mutated in Apert syndrome, where ligand specificity experiments showed that the mesenchymally expressed FGF7 is able to bind thereby to activate the mesenchymal splice form of FGFR2 (FGFR2-IIIc).

1.7. The role of the JAK-STAT signaling in human diseases

The Janus kinase (JAK)-STAT pathway plays an important role in both innate and adaptive immunity and contributes to the manifestation of several inflammatory processes. In mammals the JAK family consists of four tyrosine kinases, while the STAT family has seven members. JAK-STAT is activated by over 50 cytokines and growth factors, these cytokines include interferons, colony stimulating factors, several interleukins and hormones. Dimerized JAKs autophosphorilate and become activated enabling STAT binding and phosphorylation. Phosphorylated STAT monomers form dimers that are translocated into the nucleus of the cell and influence transcription of several genes. Several studies revealed alterations in the JAK-STAT signaling in chronic skin diseases including psoriasis, atopic dermatitis and melanoma.

STAT1 was observed to be localized predominantly in the horny layer in normal skin by immunostaining. Impaired phosphorylation of STAT1 and STAT5 was described to cause defective response to IL-2 in melanoma from stage III to IV. STAT1 in tumors functions as a suppressor probably by mediating the TNFα triggered apoptotic program. Patients with STAT1 loss-of-function mutations are more prone to mycobacterial and viral infections, whereas gain-of-function mutations increase susceptibility to fungal infections.

2. AIMS

The pathogenesis of psoriasis is still not fully understood, numerous studies have proposed hypotheses regarding to immune dysregulations, skin microbiota system or extracellular matrix alterations among others to play essential roles in the development of the disease. A continuously growing body of evidence indicate that the healthy looking NL psoriatic skin represents an unbalanced phenotype and carries structural alterations compared to normal skin making it susceptible for developing the disease. Abnormalities of the basement membrane at the dermal-epidermal junction (DEJ) render the uninvolved skin to become activated and present a chronic wound healing phenotype.

FN is capable to enhance cell cycle entry, without the increase of adhesion, in uninvolved, but not normal keratinocytes, even in the absence of T cell lymphokines. The main FN receptor α5β1 integrin was found to be overexpressed in NL psoriatic epidermis relative to normal epidermis. KGF together with fibronectin and α5β1 integrin was suggested to play a crucial role in the pathogenesis of psoriasis by influencing inflammation and keratinocyte hyperproliferation. We focus in particular on alterations in NL skin and the role of EDA^+FN and KGF in the initiation and progression of psoriasis.

Therefore, we aimed:

- To investigate the expression of KGF, FGFR2, EDA^+FN and α5 integrin in healthy and NL psoriatic skin.
- To examine whether KGF has an effect on the EDA^+FN and FN production in cultured fibroblasts, keratinocytes and HaCaT cells.
- To determine the expression of different splice forms of FGFR2 in fibroblasts, keratinocytes and melanocytes.
- To identify the signal transduction pathway(s) through which KGF may influence and modulate the expression of EDA^+FN and FN in healthy and NL skin derived fibroblasts.
- To study the putative correlation between the activation of STAT1 in healthy, NL and lesional skin and PASI score of patients.
- To construct an *in silico* model based on transcriptional regulatory molecules combined with our *in vitro* results involving KGF and FN signaling.

3. MATERIALS AND METHODS

3.1. Skin biopsies

Samples were collected from 16 patients and 25 healthy volunteers for the experiments. Mechanical stress was induced by tape-stripping procedure, and then punch biopsies were taken from NL skin of psoriatic patients (n=6) and from healthy (n=6) subjects from tape-stripped and non-treated skin area from the buttock area at 24 and 48 hours after treatment for all other experiments tissues without the introduction of mechanical stress were used.

3.2. Cell culture

Primary keratinocytes, melanocytes and fibroblasts were obtained from healthy participants or psoriatic patients by enzymatic digestion of skin biopsies according to a standard protocol. Fibroblasts, keratinocytes, melanocytes, and HaCaT cells were cultured at 37°C and 5% CO_2 in humidified standard conditions.

Fibroblasts, keratinocytes and HaCaT cells were cultured in 6-well plates at a starting density of 200,000 cells/well and incubated with 25 ng/mL KGF for 24 h; untreated cells were used as controls. For KGF signaling pathway blocking experiments the following inhibitors were used: MEK1 inhibitor, AKT ½ kinase inhibitor, STAT1 inhibitor and STAT3 inhibitor, concentrations were applied as previously described.

3.3. RNA extraction and real-time quantitative reverse transcription polymerase chain reaction (RT qRT-PCR)

Cells on 6 well plates were washed once with PBS, then harvested with 0.5 ml TRIzol™ Reagent. Total RNA was isolated according to the manufacturer`s instructions. RNA concentration was determined from A260 values. All RNA was treated with DNA-free™ reagents. cDNA was synthesized from 1 µg total RNA with the iScript cDNA Synthesis Kit according to the manufacturer's protocol. The abundance of each gene products was normalized to the 18S rRNA gene expression in each examined sample. Relative mRNA levels were calculated by the $2^{-\Delta\Delta Ct}$ method. Data are expressed as arbitrary units proportional to the mRNA level.

3.4. Immunofluorescent staining

Human punch biopsies were frozen embedded, stored at -80 °C, then cut into 6 μm sections. The primary antibodies were used: FGFR2, KGF, α_5 integrin, EDA^+FN, and pSTAT1(Ser727), pSTAT1 (Tyr701) overnight at 4°C. Goat anti-rabbit IgG-Alexa Fluor 488, donkey anti-goat IgG-Alexa Fluor 546, goat anti-mouse IgG-Alexa Fluor 647 and Alexa Fluor 546 were used as secondary antibodies for 2 h, at room temperature. Sections were incubated without the primary antibody or with isotype control antibody for negative staining controls. Nuclei were stained with DAPI.

3.5. Flow cytometry

Cells were harvested as described, above fixed in Fixation/Permeabilization Concentrate and Diluent and resuspended in PBS. Primary antibodies anti-EDA^+FN, anti-FN were applied for 45 min. Mouse IgG1 isotype antibody was used, for negative as control. Cells were then washed in PBS, and incubated with secondary antibodies as described above. Samples were analysed on a FACS Calibur flow cytometer equipped with 488 and 633 nm lasers.

3.7. Bioinformatics analysis and model construction

STRING, KEGG database and Reactome were used as a source to create protein-protein interactions and signaling pathways; SABiosciences' transcription factor binding site data and CisRED ($p<0.001$) were applied for modeling protein-DNA interactions. Direction of transcriptional regulation was assessed with expression correlation matrix. Data of a microarray study with 48 dermal fibroblast samples carried out by Gabig-Cimińska et al. was downloaded from Gene Expression Omnibus. Probe with the largest interquartile range (IQR) was selected for genes with multiple probes. Coexpressional matrix was constructed using MATLAB R2014b. The genes and proteins related to KGF signaling, fibronectin-signaling, splicing and the regulation of these genes were collected and used for filtered matrix construction.

4. RESULTS

4.1. Altered protein expression of α_5 integrin, EDA$^+$FN, KGF and FGFR2 in psoriatic NL skin

Healthy skin and psoriatic NL skin were investigated for differences in the expressions of α_5 integrin, EDA$^+$FN, KGF and FGFR2 before and 24, 48 hours after tape stripping. Immunofluorescent staining revealed a striking difference in all protein expressions examined even without tape stripping between normal and psoriatic NL skin. Mild mechanical stress introduced by tape stripping of the skin did not lead to any obvious changes in α_5 integrin, EDA$^+$FN, KGF and FGFR2 expressions or distributions at 24 and 48 hours after treatment in NL skin. In healthy control sections a slight increase in all protein expression was observed based on immunostaining.

4.2. KGF treatment of healthy human fibroblasts leads to the elevation of EDA$^+$FN production

EDA$^+$FN and KGF are known to stimulate keratinocyte proliferation. KGF induces the expression of the major FN cell surface receptor $\alpha_5\beta_1$ integrin. Given the differences in EDA$^+$FN and KGF protein levels in healthy and NL psoriatic skin, we set out to investigate a putative regulatory connection between these two molecules. Exogenous treatment of cultured healthy fibroblasts with KGF for 24 hours increased the level of the EDA$^+$FN splice variant, but not the total FN protein based on immunostaining and flow cytometry measurements. A similar increase in the EDA$^+$FN mRNA level was detected, while the total FN mRNA remained unchanged. We also investigated this effect of KGF on normal human keratinocytes and on the keratinocyte cell line, HaCaT. As expected, keratinocytes and HaCaT cells expressed the FN and the EDA$^+$FN at a very low level compared to fibroblasts. However, upon *in vitro* KGF treatment these cells did not display detectable changes in mRNA and protein expressions of EDA$^+$FN and total FN.

4.3. Expression of FGFR2 splice variants in fibroblasts, melanocytes and keratinocytes

Two splice variants have been identified for FGFR2 designated as FGFR2-IIIb and FGFR2-IIIc that have different ligand binding preferences. These results were based on mitotic

activity measurement. KGF has been shown to act on the FGFR2-IIIb variant increasing cellular proliferation. We determined the expression of the splice variants in healthy fibroblasts, melanocytes and keratinocytes using specific primers designed for RT-PCR, revealing that melanocytes and keratinocytes expressed the FGFR2-IIIb only, while fibroblasts mainly expressed the FGFR2-IIIc variant.

4.4. MAPK signaling is involved in the regulation of EDA$^+$FN controlled by KGF in healthy fibroblasts

FGF signals (including KGF) are coordinated by four major pathways: the RAS-RAF-MAPK, the PI3-AKT, the STAT and the PLCγ signaling. In order to get a better insight into the modulation of FN expression by KGF in cultured fibroblasts derived from healthy skin, we performed blocking experiments targeting key molecules of the signal transduction pathways using specific inhibitors available for MEK1 (MAPK), AKT1/2 (PI3-AKT), STAT1 and STAT3 (STAT) either alone or in combination. After 24h of inhibitory treatment the expressions of FN and EDA$^+$FN were determined by flow cytometry and RT-PCR. The KGF induced EDA$^+$FN elevation was abolished by the inhibition of MEK1 and returned to the level of control fibroblasts. In contrast, blocking AKT1/2 did not have an effect on EDA$^+$FN protein expression. The inhibition of STAT1 or STAT3 did not influence KGF-mediated changes in EDA$^+$FN levels, however, impairing these signaling molecules resulted in a KGF independent elevation of both FN and EDA$^+$FN protein levels that was significant in the case of STAT1 inhibition.

4.5. Abnormal STAT1 activation in psoriatic skin plays a role in the regulation of both FN and EDA$^+$FN

To investigate further, we compared FN and EDA$^+$FN expressions between derived from fibroblasts healthy and psoriatic NL skin 24 hours after STAT1 or STAT3 inhibition in culture. In contrast to healthy controls, blockade of STAT1 did not lead to the elevation of total FN and the EDA$^+$FN splice variant in fibroblasts from NL skin changes upon STAT3 impairment were not significant.

Therefore, we investigated the phosphorylation pattern of STAT1 at Tyr701 and Ser727 positions that are known to be key amino acid modifications leading to dimerization and influencing activity. Immunofluorescent staining for the phosphorylated Ser727 was highest in lesional psoriatic skin, lower, but clearly detectable in healthy skin, whereas NL skin samples

showed the lowest intensity. In two out of four investigated NL samples phosphorylation was undetectable. Staining for the phosphorylated Tyr701 showed a much less noticeable but similar pattern. In samples of patients where no serine phosphorylation was detected in the NL skin, the staining for the phosphorylated Tyr701 was also not visible in either NL or lesional areas. To elucidate whether differences observed in STAT1 activity among patients correlated with the severity of the disease, PASI scores of donor patients were compared. The patients whose NL skin was negative for phosphorylations of STAT1 had a lower PASI score (12.4 and 17.8), whereas low STAT1 activity in NL skin was associated with a higher PASI score (19.6 and 20).

Immunhistochemistry analysis revealed the presence of pSTAT1 (Ser727) in the both the epidermis and the dermis. STAT1 activity showed a strong correlation with PASI score. High PASI scores (eg. 19.6) were typically coupled with intensive pSTAT1 (Ser727) staining in the lesional skin and in these patients, the NL skin also showed a slight STAT1 activity. In contrast, patients with low PASI score (eg. 9.8) nearly no STAT1 activity was detectable in the NL skin. Indicating that pSTAT1 (Ser727) activation of the NL skin and the PASI scores of patients decreased in parallel. Activation of STAT1 Tyr (701) form decays rapidly and only in samples obtained from patient with high PASI score can be observed, where the presence of both phosphorylated forms is much more intensive. We compared NL skin samples near and far from the lesion for the extent of activation for the two different pSTAT1 forms in order to test whether the lesional area has an effect on surrounding NL skin. It was assessed that STAT1 activity is independent from the distance from the lesion indicating a sharp border between the NL and lesional skin. The strongest STAT1 activation is detected in psoriatic L skin.

4.6. *In silico* model construction

We constructed an *in silico* model based on our *in vitro* results involving KGF- and FN-signaling and the underlying transcriptional regulation network. Our data already suggested the participation of MAPK signaling in KGF induced FN splicing. The generated co-expression matrix implied a potential role of peptidyl-prolyl cis–trans isomerase (PPIG), a protein important in both protein folding and splicing, which is regulated by MEK1 induced AP-1. Changes in MEK1 activity could lead to the modulation of FN splicing through PPIG resulting in elevated $EDA^{+}FN$ levels. Our model also indicated that STAT3 negatively regulates the expression of MEK1. This suggested inhibitory effect of STAT3 on MEK1 may account for the increased $EDA^{+}FN$ production upon STAT inhibition.

5. SUMMARY

In psoriasis the NL skin already contains abnormalities that are likely to serve as a microenvironment favouring the manifestation of the disease. FN and its splice variant the EDA^+FN are essential extracellular matrix proteins influencing major cellular processes and they are abnormally expressed in psoriatic skin. KGF is overexpressed in psoriatic lesional skin and contribute to keratinocyte hyperproliferation. Given their function, these molecules are likely to play a part in the pathomechanism of psoriasis, therefore, in this study we focused on investigating the production of KGF, FGFR2, FN and EDA^+FN in healthy and NL psoriatic skin and studied the regulatory mechanisms involving KGF, FN and EDA^+FN in fibroblasts.

We observed that the psoriatic NL skin displays an overexpression of KGF, FGFR2, α_5 integrin and EDA^+FN compared to healthy skin. KGF mildly induced only EDA^+FN, but not FN expression in healthy fibroblasts. Our results revealed that KGF regulated EDA^+FN production takes place in an autocrine manner through MAPK signaling. Based on these results we designed an *in silico* model to predict key players in a putative regulatory network explaining the effect of KGF on EDA^+FN production. We provide *in vitro* evidence that STAT1 negatively regulates both FN and EDA^+FN expressions in healthy fibroblasts and this regulation is compromised in fibroblasts derived from NL psoriatic dermis. We detected active STAT1 in healthy and lesional skin, as reported previously, however, in the NL skin STAT1 activation was close to absent in samples irrespectively from the distance from lesions.

These result led us to the conclusion that the production of FN, EDA^+FN by fibroblasts and the signaling of STAT1 is abnormally regulated in the psoriatic NL skin, suggesting a crucial mechanism for keeping NL skin in a preactivated state for developing a chronic wound healing phenotype.

LIST OF PUBLICATION

Publications directly related to the subject of the dissertation

I. Guban B, Vas K, Balog Z, Manczinger M, Bebes A, Groma G, Szell M, Kemeny L, Bata- Csorgo Zs
Abnormal regulation of fibronectin production by fibroblasts in psoriasis. Br J Dermatol. 2016 Mar; 174(3):533-541. doi: 10.1111/bjd.14219. Epub 2016 Jan 3.
IF: 4.275

II. Gubán B, Kui R, Képíró L, Bebes A, Groma G, Kemény L, Bata-Csörgő Zs
Abnormal STAT1 activation in psoriasis
Hungarian Journal of Dermatology and Venerology Review, 2016 February, 92:18-21. doi: 10.7188/bvsz.2016.92.1.3.

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